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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : C07H 21/00, 21/04	A1	(11) International Publication Number: WO 94/24143 (43) International Publication Date: 27 October 1994 (27.10.94)
(21) International Application Number: PCT/US94/03747 (22) International Filing Date: 6 April 1994 (06.04.94) (30) Priority Data: 08/046,032 12 April 1993 (12.04.93) US (71) Applicant: NORTHWESTERN UNIVERSITY [US/US]; 633 Clark Street, Evanston, IL 60208-1111 (US). (72) Inventors: LETSINGER, Robert, L.; 316 3rd Street, Wilmette, IL 60091 (US). GRYAZNOV, Sergei, M.; 2 Clark Drive, San Mateo, CA 94401 (US). (74) Agent: KOHN, Kenneth, L.; P.O. Box 4390, Troy, MI 48099 (US).		(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>
(54) Title: METHOD OF FORMING OLIGONUCLEOTIDES (57) Abstract A method of forming an oligonucleotide is disclosed, the method including the steps of disposing in solution at least two oligonucleotides in aqueous solution wherein one of the oligonucleotides includes an α -haloacyl group and the other of the nucleotides includes a phosphothioate group and covalently binding the oligonucleotides together through the α -haloacyl group and the phosphothioate group spontaneously forming a thiophosphorylacetylamino group therebetween.		

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METHOD OF FORMING OLIGONUCLEOTIDES

TECHNICAL FIELD

The present invention relates to a
5 method of forming oligonucleotides and more
specifically to methods having use as potential
new therapeutic methods for treating viral
diseases, cancer, genetic disorders and the like,
as well as diagnostic applications of
10 oligonucleotides.

BACKGROUND OF THE INVENTION

Antisense oligonucleotides have
demonstrated potential as new types of
15 therapeutic agents for treating such diseases and
disorders as viral diseases, cancer, genetic
disorders, as well as other diseases and
disorders¹. Extensive research has been carried
out and is being continued in industrial and
20 academic laboratories to explore this potential².

A problem that has been encountered
with the approach of utilizing antisense
oligonucleotides as therapeutic agents is related
to the selectivity of the agents *in vivo*. In
25 view of the low concentrations of intracellular
polynucleotide targets and the low concentrations

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of therapeutic oligonucleotides that can be introduced into cells, it is recognized that there is a need for oligonucleotides with high binding affinities. The binding affinity is
5 related to the length of the oligonucleotides, preferably 20-mers and longer. But, in the case of long oligonucleotides, a mismatch in base pairing is less destabilizing than in the case of a short oligonucleotide. Hence, the desired
10 destabilizing effect is lessened by the use of longer oligonucleotides while the selectivity is increased.

Experts have noted that "high sequence specificity" and "high affinity" are
15 contradictory demands³. It has further been concluded that on the basis of the extent to which antisense oligonucleotides can cause cleavage of RNAs at imperfectly matched target sites, in systems that were tested it was
20 probably not possible to obtain specific cleavage of an intended target RNA without also causing at least the partial destruction of many non-targeted RNAs⁴. Hence, experts in the field, based on conducted research, have concluded that
25 the conflicting requirements of specificity and affinity are major hurdles to overcome.

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Several methods have been reported for covalently linking oligonucleotide blocks in aqueous media^{5a-1}. All of these methods require an additional chemical agent to yield a stable
5 ligated product. Depending on the approach, the added reagent may be an "activating agent" such as a water soluble carbodiimide or cyanoimidazole^{5a-k} or it may be a reducing agent such as sodium cyanoborohydride^{5l}. In either
10 case, the need for the third reagent precludes chemical ligation *in vivo* since such compounds are toxic, react with water, and could not be introduced into living systems in sufficient amounts to bring about the desired coupling
15 reaction.

The present invention provides a novel method for covalently linking oligonucleotide blocks present in low concentrations in an aqueous medium without need for an additional
20 condensing or stabilizing reagent. It therefore opens the door for *in situ* chemical ligation in living systems. Since the reactions are greatly accelerated in the presence of a complementary oligonucleotide sequence, one should in principle
25 be able to form long oligonucleotide strands selectively *in vivo* when a target polynucleotide

(e.g. m-RNA or DNA from a virus or cancer cell) containing consecutive nucleotide sequences complementary to the individual oligonucleotide strands is present. Long oligonucleotide
5 strands, which bind with high affinity, would therefore be generated *in situ* from shorter strands that bind with lower affinity, when the target polynucleotide is present. This invention could therefore solve the problem of the conflict
10 of achieving high affinity as well as high specificity, in therapeutic and also in diagnostic applications.

SUMMARY OF THE INVENTION

15 In accordance with the present invention there is provided a method of forming an oligonucleotide by irreversibly covalently linking at least two oligomers which themselves are reversibly bound by hydrogen bonding at
20 adjacent positions on a target polynucleotide containing a nucleoside base sequence complementary to the sequences of the pair of oligomers, wherein one of the oligonucleotides includes a nucleotide having a first reactive
25 group adjacent to a nucleotide of the other oligomer which includes a second reactive group

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capable of spontaneously forming a covalent bond with the first reactive group. The oligonucleotides are covalently joined together through the first and second reactive groups
5 having been brought into proximity to each other upon binding of the oligonucleotides on the polynucleotide.

The present invention further provides a method of forming an oligonucleotide by
10 disposing at least two oligonucleotides in aqueous solution wherein one of the oligonucleotides includes an α -haloacyl group and the other nucleotide includes a phosphothioate group. The oligonucleotides are covalently bound
15 together through the α -haloacyl group and the phosphothioate group spontaneously forming a thiophosphorylacetylamino group therebetween.

BRIEF DESCRIPTION OF THE FIGURES

20 Other advantages of the present invention will be readily appreciated as the same becomes better understood by reference to the following detailed description when considered in connection with the accompanying drawings
25 wherein:

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Figure 1 shows the coupling of two short oligomers in accordance with the present invention utilizing a target template;

Figure 2 shows the facile reaction of
5 an oligonucleotide phosphorothioate with an α -haloacyl oligonucleotide derivative in accordance with the present invention;

Figure 3 shows results of ion exchange high performance liquid chromatography (IE HPLC)
10 of products from experiment 1 wherein: A, after 2 hours in solution at 0°C; B, after 2 days at 0°C; and C, after the final step in which the solution was frozen and stored at -18°C for 5 days, the peaks at approximately 17, 21 and 24 minutes
15 correspond to compounds 1, 2, and 3, respectively.

Figure 4 shows IE HPLC of products from a second experiment (frozen, -18°C throughout) after: wherein A, after 2 hours in solution at
20 0°C; B, after 2 days at 0°C; and C, after: A, 5 hours; B, 2 days; and C, 5 days, the peaks at approximately 17, 21, and 24 minutes corresponding to compounds 1, 2, and 3, the peak at 27 minutes corresponding to the dimer
25 derivative of compound 2 produced by oxidation by air; and

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Figure 5 shows the following: A, IE
HPLC of products from the reaction of compounds 1
and 2 in presence of template 4 at 0°C after 2
hours, the major peaks corresponding to coupling
5 product 3 and template 4, noting that compound 1
(peak at 17 minutes) has been almost completely
consumed; B, same products after treatment with
KI₃ followed by Dithiothreitol (DTT); noting that
compound 3 has been replaced by two
10 oligonucleotide cleavage products, eluting at 18
and 22 minutes.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present
15 invention there is provided a method of forming
an oligonucleotide generally by the steps of
disposing at least two oligonucleotides in
aqueous solution wherein one of the
oligonucleotides includes an α -haloacyl group and
20 the other of the nucleotides includes a
phosphothioate group and then covalently binding
the oligonucleotides together through the α -
haloacyl group and the phosphothioate groups
spontaneously forming a thiophosphorylacetyl amino
25 group therebetween.

This method exploits the fact that the coupling reaction described herein is very slow in very dilute aqueous solutions but is fast in the presence of a template polynucleotide. That is, the reaction is accelerated in the presence of a target polynucleotide that possesses the sequence section complementary to the probe oligomers. The present invention employs as a therapeutic agent two short oligomers (for example, 8 to 20-mers) which will spontaneously link together covalently after binding at adjacent positions on the target polynucleotide. With this system, one will approach the binding affinity and recognition properties of a longer oligomer probe such as between 16 to 40-mer, but retain the dependency and base pairing characteristics of the shorter probes (8 to 20-mer). In other words, the present invention provides the specificity of shorter polynucleotides while possessing the effect of longer polynucleotides.

Inherent in the present invention is the need and use of polynucleotides including reactive groups which will spontaneously react to form a covalent bond therebetween when the groups are in spacial proximity to each other.

Specifically, the present invention utilizes at least two oligonucleotides wherein one set of oligonucleotides includes the first reactive group and the second set of oligonucleotides include the second reactive group such that upon being brought in proximity to each other, the groups will spontaneously react to form a stable covalent bond. Examples of such pairs of reactive groups are ester+hydrazide, $RC(O)S^-$ +haloalkyl and RCH_2S^- + α -haloacyl. Preferably, the present invention utilizes an α -haloacyl group, such as a bromoacetyl amino group and a thiophosphoryl group, which form a thiophosphorylacetyl amino bridge efficiently, selectively, and irreversibly in dilute aqueous media. As demonstrated below, the products are stable in water and hybridize well with complementary polynucleotides.

At low oligomer concentrations, such as less than $1\ \mu M$, and in absence of a complementary template the reactions are very slow but can be carried out to high conversion within a few days by freezing the solution. The freezing techniques are described in detail below.

Coupling is quite fast (greater than 90% conversion in 20 minutes) when carried out in

solution in the presence of a complementary oligonucleotide that serves as a template, as shown below in the Example section.

Selectivity is also a major concern in
5 diagnostic applications of the present invention and generally in the use of oligonucleotides. The same features of the present invention that make the novel chemistry of the present invention attractive for therapeutic applications also make
10 it attractive for diagnostic uses. For example, the present invention could be utilized in a diagnostic system as follows.

Referring to Figure 1, A is an oligomer consisting of, for example, a 10-mer bearing a
15 marker (*) in the chain and a bromoacetyl amino group at the 3'-terminus. B is another short oligomer with a thiophosphoryl group at the 5' end. C is a target oligonucleotide sequence with a sequence complementary to A + B. If in dilute
20 solution the coupling of A and B is sufficiently slow in absence of the template, relative to coupling in the presence of the template, only coupling on the template will be significant. This chemical ligation system could therefore be
25 employed in amplification and detection analogously to the enzymatic ligation system

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(Ligase Chain Reaction, or LCR). It has the potential to be superior since some non-specific coupling introduces a source of error in the enzymatic scheme. The fact that at very low concentrations of oligonucleotides (that is, in the range of interest in diagnostic applications) the rate of the chemical ligation in absence of template becomes extremely slow indicates that the non-template directed coupling could be unimportant in this case.

EXAMPLES

As shown in Figure 2, the ligation indicated in equation 1 for oligomers 1 and 2 exploits the facile reaction of a phosphorothioate with an α -haloacyl derivative.

Specifically, compound 1 (Seq. ID 1) in Figure 2 has a 3'-(bromoacetyl amino)-3'-deoxythymidine unit at the 3'-terminus. For preparation of compound 1, 15 μ L of 0.4 M aqueous N-succinimidyl bromoacetate (obtained from Calbiochem) was added to 4.9 A_{260} units of the 3'-aminodeoxyribo-oligonucleotide precursor, ACACCCAATT-NH₂. The method of preparation is described by Gryaznov et al., 1992⁶. The reaction was carried out in 10 μ L of 0.2 M sodium

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borate buffer at room temperature. After 35 minutes, the mixture was diluted with 0.5 mL of water, desalted by gel filtration on a NAP-5 column (produced by Pharmacia), and purified by
5 RP HPLC high pressure liquid chromatography and again desalted, giving 4 A₂₆₀ units of compound 1. The elution times are as follows: RP HPLC, 17.4 minutes; IE HPLC, 17.4 minutes.

The IE HPLC carried out above and all
10 similar procedures carried out below was carried out on a Dionex Omni Pak NA100 4x250 mm column at pH 12 (10 mM sodium hydroxide) with a 2% per minute gradient of 1.0 M sodium chloride in 10 M sodium hydroxide. For RP HPLC, a Hypersil ODS
15 column (4.6x200mm) was used with a 1% per minute gradient of acetonitrile in 0.03 M triethylammonium acetate buffer at pH 7.0.

Compound 2 (Seq. ID 2) was synthesized on a 1 μ mole scale on a Milligen/Bioscience
20 Cyclone DNA Synthesizer using LCAA CPG supported 5'-dimethoxytrityl-N-isobutyryldeoxyguanosine. Standard cyanoethyl phosphoramidite chemistry was used. When chain elongation was complete, the terminal 5'-hydroxyl group was phosphitilated (5
25 minutes) with 150 μ L of a 0.1 M solution of "Phosphate ONTM" reagent (from Cruachem) in

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acetonitrile and 150 μ L of 0.5 M tetrazole in acetonitrile. The resulting phosphite was sulfurized by treatment with a 5% solution of sulfur in pyridine/carbon disulfide (1:1, v/v, 45 minutes at room temperature). After cleavage of the DMT group (3% DCA in dichloromethane, 1.5 minutes) the supported polymer was worked up as in the case of compound 1.

Reaction of a thiophosphoryl-oligonucleotide with a haloacetylaminaromatic derivative in DMS and water has been employed in preparing dye-oligonucleotide conjugates⁷.

Depending upon the use of the invention and the desired kinetics, coupling of the oligonucleotides can be carried out in either aqueous solution, in a frozen state in ice, or in an aqueous solution in the presence of template, as discussed above and as exemplified below.

In an initial experiment, 1.0 mL of a solution (pH 7.05, 15 mM phosphate, 85 mM NaCl) containing compounds 1 (0.39 A_{260} units, 4 μ M) and 2 (0.41 A_{260} units, 4 μ M) was prepared and kept at 0°C for 5 days. The solution was warmed to 50°C for 2.5 hours, and finally frozen and stored at -18°C for an additional 5 days. Analysis by IE HPLC of samples after 2 hours and 48 hours showed

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formation of a slower eluting product, oligomer 3 (Figure 2), in yields of about 25% and 80%, respectively. No significant change was observed after the additional 3 days at 0°C or warming at 50°C. However, the reaction did proceed further in the frozen state, affording a high conversion to compound 3 (Seq. ID 3) within 5 days as shown in Figure 3. The enhanced extent of reaction in the ice matrix may be attributed to the high local concentration of the oligonucleotide reactants within the cavities in the ice. Other reactions have been similarly carried out in an ice matrix⁸.

In light of this result, an equimolar mixture of compounds 1 and 2 (2 μ M each) in the same buffer was directly frozen and held at -18°C. The HPLC profiles obtained from samples after 5 hours and daily thereafter show progression to give a high yield of 3 in 5 days, Figure 4 showing representative data.

Data for coupling compounds 1 and 2 in solution in the presence of a complementary oligonucleotide template (CCATTTTCAGAATTGGGTGT, compound 4 (Seq. ID 4)) are presented in Figure 5. The system was the same as in the first experiment except template 4 was also present (4

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μM). In this case the reaction proceeded to >90% completion within 20 minutes and was essentially complete within 2 hours.

The structure assigned to compound 3 is supported by the properties of a model compound (T-NHC(O)CH₂-SP(O)(O⁻)O-T, prepared in solution on a larger scale than used for compound 3), by the mobility of compound 3 on gel electrophoresis (R_m 0.58, compared to R_m 0.89, 0.95, and 0.61 for compounds 1, 2, and 4, respectively), and by the stability of the complex formed with the complementary oligonucleotide, 4. Retention time, RP HPLC 10.5 minutes; FAB⁺ mass spectrum, M+H⁺ 620, M+Na⁺ 642; ³¹P NMR, δ in D₂O, 18.7 ppm, prior references have disclosed characteristics for the alkylthiophosphate group.⁹

R_m values are relative to bromophenol blue in a 20% polyacrylamide/5% bis acrylamide gel. The T_m value, 56°C in 0.1 M NaCl, approaches that of the complex formed from the corresponding all-phosphodiester 20-mer and compound 4 (60°C)¹⁰ and differs significantly from values for complexes formed from compounds 1 or 2 with compound 4 (37°C and 31°C). In addition, the internucleotide -NH(CO)CH₂SP(O)(O⁻)- link was found to be cleaved selectively on oxidation with

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KI₃⁹ (Figure 5). More specifically, the duplex containing compounds 3 and 4 (0.3 A₂₆₀ units each) in 100 μ L of water was treated with 100 μ L of 0.2 M aq. KI₃ for 15 minutes at 50°C. Then 10 μ L of 1
5 M aq. DTT was added to the solution. After 5 minutes the mixture was desalted on a NAP-5 column and analyzed by IE HPLC.

The above experimentation provides evidence that the present invention presents
10 novel chemistry which provides a convenient means for selectively and irreversibly coupling oligonucleotides in aqueous solution in the range of 4 μ M oligomer concentration or greater. The products have been shown to be stable in neutral
15 solution and for a few hours even at pH 12 at room temperature. At concentrations below 1 μ M, the rate in the liquid phase become extremely slow. However, the reactions can be carried to near completion in the frozen state. The rate of
20 coupling is markedly accelerated by the presence of a complementary oligonucleotide template. These properties provide a potential in the design of chemical amplification systems and *in situ* ligation in antisense application as well as
25 in building complex structures from oligonucleotide blocks based on known chemistry.

The invention has been described in an illustrative manner, and it is to be understood that the terminology which has been used is intended to be in the nature of words of
5 description rather than of limitation.

Obviously many modifications and variations of the present invention are possible in light of the above teachings.

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25

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Letsinger, Robert L.
Gryaznov, Sergei M.
- (ii) TITLE OF INVENTION: METHOD OF FORMING
OLIGONUCLEOTIDES
- (iii) NUMBER OF SEQUENCES: 4
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Reising, Ethingthon, Barnard,
Perry & Milton
 - (B) STREET: P.O. Box 4390
 - (C) CITY: Troy
 - (D) STATE: Michigan
 - (E) COUNTRY: USA
 - (F) ZIP: 48099
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 08/046,032
 - (B) FILING DATE: 12-APR-1993
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Kohn, Kenneth I.
 - (B) REGISTRATION NUMBER: 30.955
 - (C) REFERENCE/DOCKET NUMBER: NU9310
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (313) 689-3554
 - (B) TELEFAX: (313) 689-4071

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 11 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: both
- (ii) MOLECULE TYPE: DNA (genomic)
- (ix) FEATURE:
 - (A) NAME/KEY: misc difference
 - (B) LOCATION: replace(1..11, "")
 - (D) OTHER INFORMATION: /note= "N is a bromoacetylamino
group"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ACACCCAATT N

11

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 11 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: both

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: misc_difference
- (B) LOCATION: replace(1..2, "")
- (D) OTHER INFORMATION: /note= "N is a thiophosphoryl group"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

NCTGAAAATG G

11

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: both

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: misc_difference
- (B) LOCATION: replace(11..12, "")
- (D) OTHER INFORMATION: /note= "NN is a thiophosphorylacetyl amino group"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ACACCCAATT NNCTGAAAAT GG

22

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: both

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: misc feature
- (B) LOCATION: 1..20
- (D) OTHER INFORMATION: /note= "Complementary to Seq. 3
without NN"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CCATTTTCAG AATTGGGTGT

20

CLAIMSWhat is Claimed is:

1. Method of forming an
5 oligonucleotide by:
 - a) reversibly binding at least two
oligonucleotides at adjacent positions on an
oligo- or polynucleotide including base units
complementary to base units of the oligomers,
10 wherein one of the oligonucleotides includes a
nucleotide having a first reactive group
proximate to a nucleotide of the other oligomer
which includes a second reactive group capable of
spontaneously forming a covalent bond with the
15 first reactive group; and
 - b) irreversibly covalently joining the
oligonucleotides together through the first and
second reactive groups having been brought in
proximity to each other upon binding of the
20 oligonucleotides on the polynucleotide.
2. A method of forming an
oligonucleotide of claim 1 wherein the first
reactive group is an α -haloacyl and the second
25 reactive group is a phosphothioate, said step (b)
being further defined as spontaneously forming a

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thiophosphorylacetyl amino bond through the reactive groups.

3. A method of forming an
5 oligonucleotide of claim 1 wherein each of the oligomers consists of 8 to 20 nucleotides.

4. A method of forming an
oligonucleotide of claim 1 wherein steps (a) and
10 (b) occur in aqueous solution.

5. A method of forming an oligonucleotide by:

a) disposing at least two
15 oligonucleotides in aqueous solution wherein one of the oligonucleotides includes an α -haloacyl group and the other of the nucleotides includes a phosphothioate group; and

b) covalently binding the
20 oligonucleotides together through the α -haloacyl group and the phosphothioate groups spontaneously forming a thiophosphorylacetyl amino group therebetween.

6. A method of forming an oligonucleotide of claim 5 further including the step of (c) accelerating the reaction and carrying the reaction out to high completion by
- 5 freezing the aqueous solution containing the oligonucleotides therein.

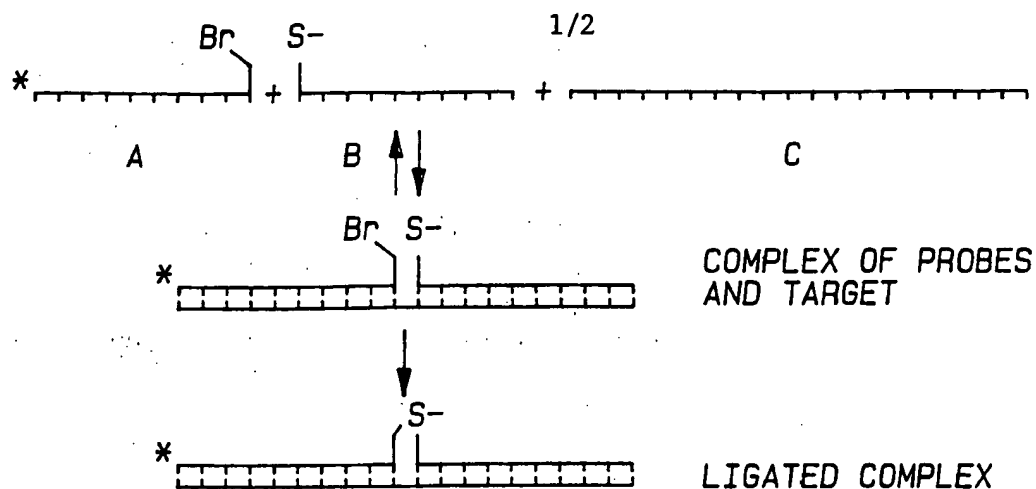


Fig-1

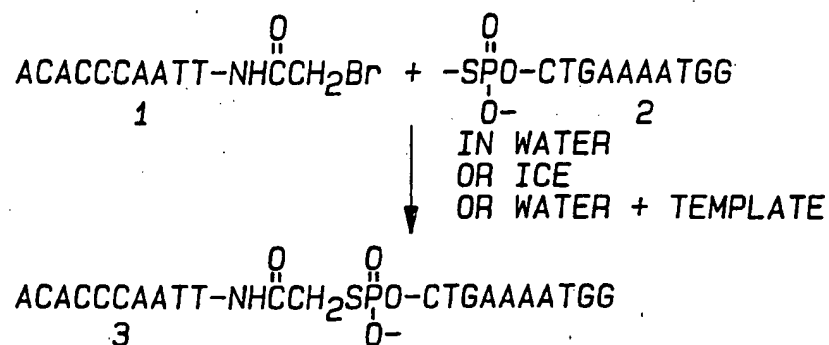


Fig-2

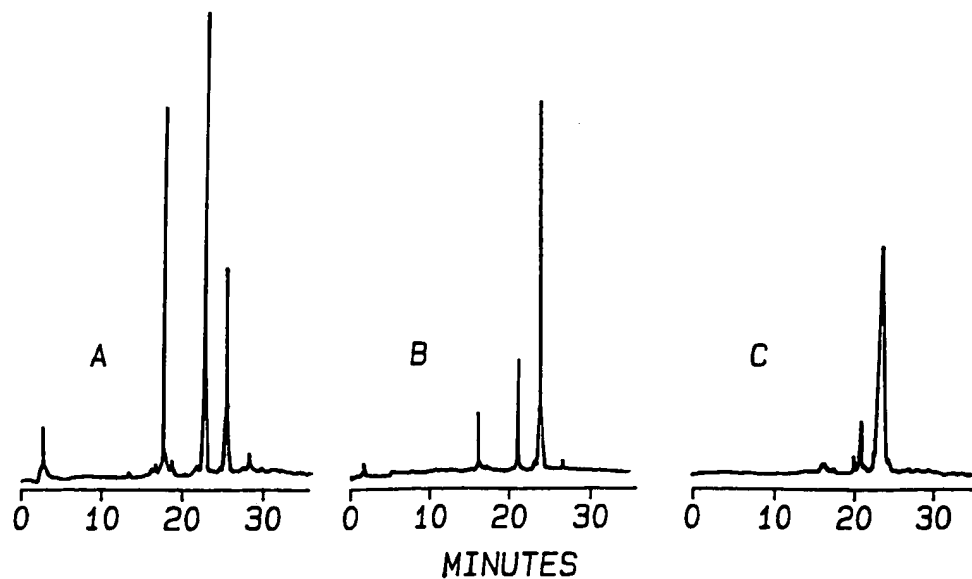


Fig-3

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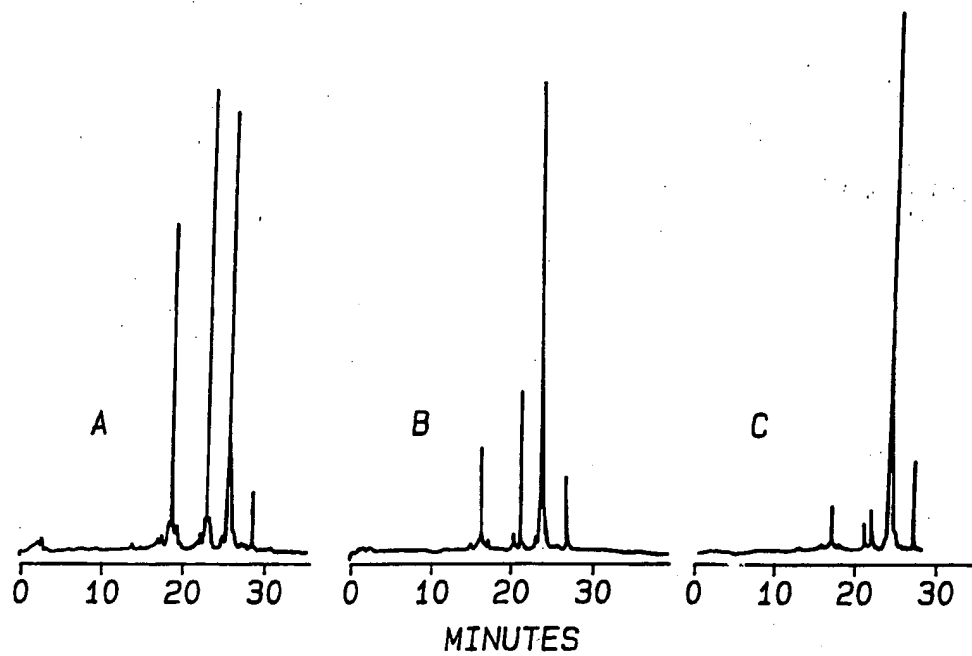


Fig-4

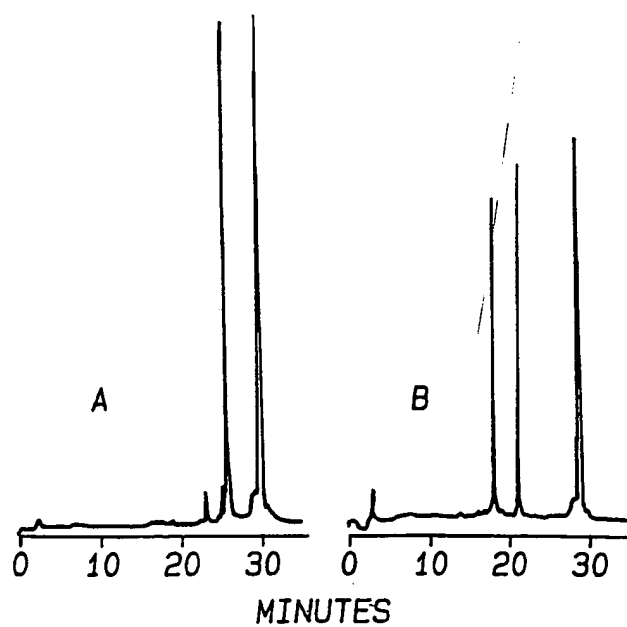


Fig-5

INTERNATIONAL SEARCH REPORT

 International application No.
 PCT/US94/03747

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : CO7H 21/00, 21/04

US CL : 536/25.3

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/25.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

NONE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	Journal of the American Chemical Society, Volume 114, issued 1992, Goodwin et al., "Template-Directed Synthesis: Use of a Reversible Reaction", pages 9197 - 9198, see entire document.	<u>1</u> 2 - 6
X	Alberts et al., "Molecular Biology of the Cell", published 1983 by Garland Publishing, Inc. (N.Y.), page 187, see entire document.	1
A	Journal of the American Chemical Society, Volume 115, issued 1993, Gryaznov et al., "Chemical Ligation of Oligonucleotides in the Presence and Absence of a Template", pages 3808 - 3809.	1 - 6



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G*	document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

22 JUNE 1994

Date of mailing of the international search report

JUL 19 1994

 Name and mailing address of the ISA/US
 Commissioner of Patents and Trademarks
 Box PCT
 Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

GARY L. KUNZ

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/03747

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Tetrahedron Letters, Volume 28, Number 36, issued 1987, Thuong et al., "Synthese et Reactivite D'oligothymidylates Substitues par un Agent Intercalant", pages 4157 - 4160, see abstract on page 4157, last sentence.	1 - 6